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# Insulin elevates leptin secretion and mRNA levels via cyclic AMP in 3T3-L1 adipocytes deprived of glucose

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## Abstract

**Aims:** Leptin plays an important role in the pathogenesis of obesity and diabetes, yet the regulatory mechanisms of this hormone have not been fully elucidated. In this study, we aimed to clarify the roles of insulin and glucose in leptin secretion and mRNA production using inhibitors of insulin signal transduction in adipocytes cultured under glucose-free or normal conditions.

**Methods:** Differentiated 3T3-L1 adipocytes were stimulated with insulin in combination with inhibitors for phosphoinositide 3-kinase (PI3K), Akt, and phosphodiesterase 3B (PDE3B), as well as epinephrine and a cyclic AMP (cAMP) analog under glucose-free or normal conditions. After 8 h of stimulation, leptin protein levels in the media and leptin mRNA expression levels in the adipocytes were measured.

**Results:** Insulin significantly increased the secretion and mRNA levels of leptin under the depletion of glucose. Glucose augmented basal leptin secretion without insulin, while glucose nullified insulin-induced leptin mRNA upregulation. The

PI3K inhibitor BEZ-235, the Akt inhibitor MK-2206, and the PDE3B inhibitor cilostazol attenuated the insulin stimulation of leptin secretion, but did not suppress the insulin-induced leptin mRNA upregulation with glucose depletion. In contrast to the glucose-free condition, insulin failed to upregulate leptin mRNA in the presence of glucose. The cAMP analog dibutyryl cAMP and epinephrine decreased both leptin secretion and mRNA regardless of glucose supplementation.

**Conclusion:** Insulin alone stimulates leptin secretion and elevates leptin mRNA levels via cAMP under the lack of glucose metabolism, while glucose is a significant and ambivalent effector on the insulin effects of leptin.

Keywords: Medicine, Endocrinology

## 1. Introduction

Leptin, a hormone secreted by mature adipocytes, plays an important role in the pathogenesis of obesity and diabetes. Leptin suppress appetite, increases energy consumption, and enhances insulin sensitivity via its receptors in the hypothalamus [1, 2, 3].

The concentration of serum leptin has been reported to correlate with body fat percentage [4], indicating that the total amount of adipocytes may be an important determinant of leptin levels. On the contrary, multiple hormones and nutrients affect leptin secretion [5, 6] and insulin is a well demonstrated secretagogue of leptin both *in vitro* and *in vivo* [7, 8, 9, 10, 11, 12, 13, 14, 15, 16]. Nevertheless, the mechanisms of insulin action on leptin are still arguable.

The insulin stimulation of leptin has been attributed to accelerated glucose metabolism rather than direct effects [12, 13, 14]. In contrast, multiple studies have suggested that insulin-stimulated leptin secretion is not dependent on glucose [15, 16, 17]. Although the phosphoinositide 3-kinase (PI3K)- phosphodiesterase 3B (PDE3B) pathway [7, 10] and cyclic AMP (cAMP) [7, 18, 19, 20] have been reported to mediate the insulin stimulation of leptin, whether the insulin signal contributes to leptin secretion via accelerated glucose metabolism or directly affects it irrespective of glucose still needs to be clarified.

It is also debatable whether insulin signals and glucose catabolism enhance only leptin secretion or also upregulate leptin synthesis. Insulin stimulation of leptin levels has been associated with its secretion, yet reported observations are diverse depending on experimental conditions [5]. Accelerated glucose metabolism may also increase leptin mRNA levels or protein synthesis in adipocytes [14, 16, 21] and inversely, deprivation of glucose may downregulate leptin mRNA levels [14], although the effects may not be specific to glucose metabolism but simply be attributed to energy status [22].

To elucidate these issues, we examined the role of insulin on the secretion and the mRNA levels of leptin in mature 3T3L1 adipocytes cultured under the depletion of glucose and under normal conditions. Here we report that glucose is not essential for insulin effects on leptin secretion and mRNA levels yet is a significant and ambivalent factor of insulin effects.

## 2. Materials and methods

### 2.1. Cell culture

The 3T3-L1 pre-adipocytes were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 25 mM glucose and 10% fetal bovine serum (FBS; Thermo Scientific, Waltham, MA, USA), and penicillin and streptomycin (Sigma-Aldrich) at 37 °C in a humidified 5% CO<sub>2</sub>/95% air atmosphere. Two days after reaching full confluence, the pre-adipocytes were differentiated into mature adipocytes by culturing with 10% FBS supplemented DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich), 0.25 μM dexamethasone (Sigma-Aldrich), and 5 μg/ml insulin (Sigma-Aldrich) for two days, and subsequently with medium containing 5 μg/ml insulin for two days. The 3T3-L1 adipocytes were used for experiments at day 10 of differentiation.

The prepared adipocytes were incubated in serum-free DMEM containing 3% BSA with glucose at concentrations of 0, 5.5 mM, or 25 mM, and in the presence or absence of insulin (0.5 μM), the PI3K inhibitor BEZ-235 (0.1–10 μM, Selleck, Houston, TX, USA), the serine/threonine protein kinase (Akt) inhibitor MK-2206 (0.1–10 μM, Cayman Chemicals, Ann Arbor, MI, USA), the phosphodiesterase 3B (PDE3B) inhibitor cilostazol (0.1–1 μM, Cayman Chemicals), a membrane-permeable cAMP analog dibutyryl cAMP (100–400 μM, R&D Systems, Minneapolis, MN, USA), epinephrine (0.1–10 μM, Sigma-Aldrich), an alpha-1 adrenergic antagonist prazosin (10 μM, Sigma-Aldrich), and a nonselective beta-blocking agent propranolol (10 μM, Sigma-Aldrich). The doses of each agent were selected in reference to previous studies [23, 24, 25, 26]. The incubation time was determined as 8 h, because time course experiment revealed that both leptin mRNA and secretion were significantly increased with insulin stimulation at 8 h, and further incubation under glucose-free and serum-free condition may damage cell functions. After incubation, cell culture media and cells were collected and used to measure leptin protein concentrations and mRNA levels.

### 2.2. Quantification of leptin

Leptin levels in media samples were measured using a sandwich enzyme-linked immunosorbent assay kit (R&D Systems) as per the manufacturer's instructions,

except that we used twice volume of samples. Intra-assay and inter-assay CV values are 4.3% and 7.6%, respectively. The sensitivity we validated was 7.8 pg/ml.

### 2.3. Quantitative real-time PCR

Total RNA was extracted from 3T3-L1 adipocytes using the High Pure RNA<sup>TM</sup> kit (Roche, Indianapolis, IN, USA) and reverse transcribed into cDNA by a PrimeScript<sup>TM</sup> RT reagent kit (Takara Bio, Kusatsu, Japan) under the conditions recommended by the manufacturer. Real-time PCR analysis was undertaken using SYBR Premix Ex Taq<sup>TM</sup> (Takara Bio) and a Step One Plus<sup>TM</sup> System (Applied Biosystems, Foster City, CA, USA). Amplification consisted of an initial step (95 °C for 30 s), 40 cycles of denaturation for 5 s at 95 °C and annealing for 30 s at 60 °C. The measurement was duplicated and the expression level of leptin mRNA was normalized relative to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The mouse leptin primers used were 5'-caggatcaatgacatttcacaca-3' and 5'-gctggtgaggacctgttgat-3' and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-tccactcatggcaaattcaa-3' and 5'-tttgatgttagtgggtctcg-3'. The mRNA expression levels of leptin were normalized relative to GAPDH mRNA levels.

### 2.4. 3-(4,5-Dimethylthiazol-2-yl)-5(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

To assess cytotoxicity by inhibitors, cells were cultured with each agent for 24 h, and 20 µL of CellTiter 96 Aqueous One Solution Reagent<sup>TM</sup> (Promega, Madison, WI) was added to each well. The plate was incubated for 1 h, followed by the reading of absorbance at 490 nm.

### 2.5. Statistical analyses

All statistical analyses were performed with the Prism 5.02 program (GraphPad Software, San Diego, CA, USA). For comparison among groups, the Tukey-Kramer method and one-way analysis of variance (ANOVA) were applied. P values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Effect of insulin and glucose on the secretion and mRNA expression of leptin in 3T3-L1 adipocytes

We first examined the effects of insulin on leptin secretion and mRNA expression in the absence or presence of glucose in 3T3-L1 adipocytes. Insulin significantly increased both secretion and mRNA expression of leptin under the depletion of

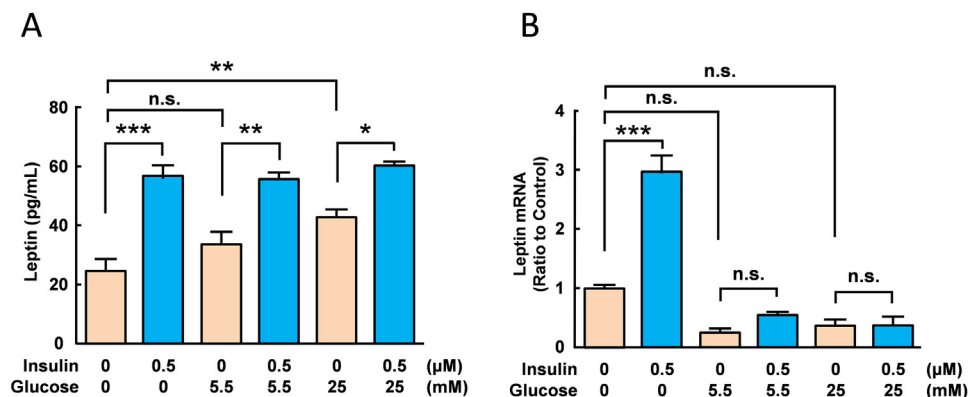
ambient glucose (Fig. 1A and B). Glucose augmented basal leptin secretion without insulin stimulation in a concentration-dependent manner, whereas glucose did not amplify the insulin effects on leptin secretion. Neither 5.5 mM nor 25 mM glucose increased mRNA expression of leptin, and the insulin stimulation on leptin mRNA seen in glucose-free media was cancelled in the presence of glucose (Fig. 1B).

### 3.2. Regulation of leptin secretion and mRNA expression by insulin via the PI3K-AKT pathway with the depletion of glucose

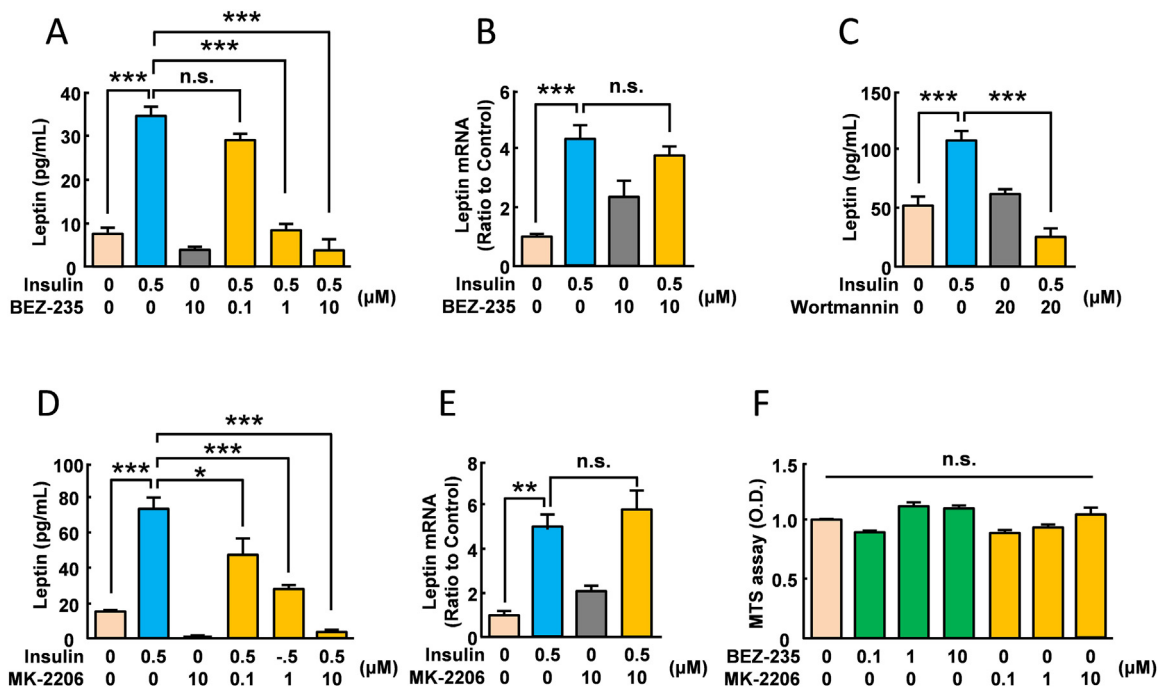
We then examined the effects of insulin signal inhibitors on leptin secretion and mRNA levels in 3T3-L1 adipocytes deprived of glucose. The PI3K inhibitor BEZ-235 attenuated the insulin stimulation of leptin secretion in a dose-dependent manner (Fig. 2A). In contrast, BEZ-235 did not suppress the upregulation of leptin mRNA by insulin (Fig. 2B). Another PI3K inhibitor, wortmannin, also inhibited the insulin-induced leptin secretion (Fig. 2C). The effects of the Akt inhibitor MK-2206 were similar. MK-2206 dose-dependently inhibited insulin-induced leptin secretion but not its mRNA expression with glucose depletion (Fig. 2D and E). Neither BEZ-235 nor MK-2206 affected cell viability assessed by MTS assay (Fig. 2F).

### 3.3. Effects of PDE3B inhibition and cAMP on leptin secretion and mRNA expression

PDE3B, an enzyme catalyzing the conversion of cAMP to AMP, is expressed in adipocytes [7] and located downstream of Akt signaling. The PDE3B inhibitor cilostazol suppressed insulin stimulated leptin secretion in a dose-dependent manner, while it showed no effect on leptin mRNA levels under the lack of glucose



**Fig. 1.** Effects of glucose concentration and insulin on the secretion (A) and mRNA levels (B) of leptin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 8 h in DMEM containing 0, 5.5, or 25 mM glucose in the presence or absence of 0.5 μM insulin. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . n.s., not significant. Data are expressed as means  $\pm$  SE.

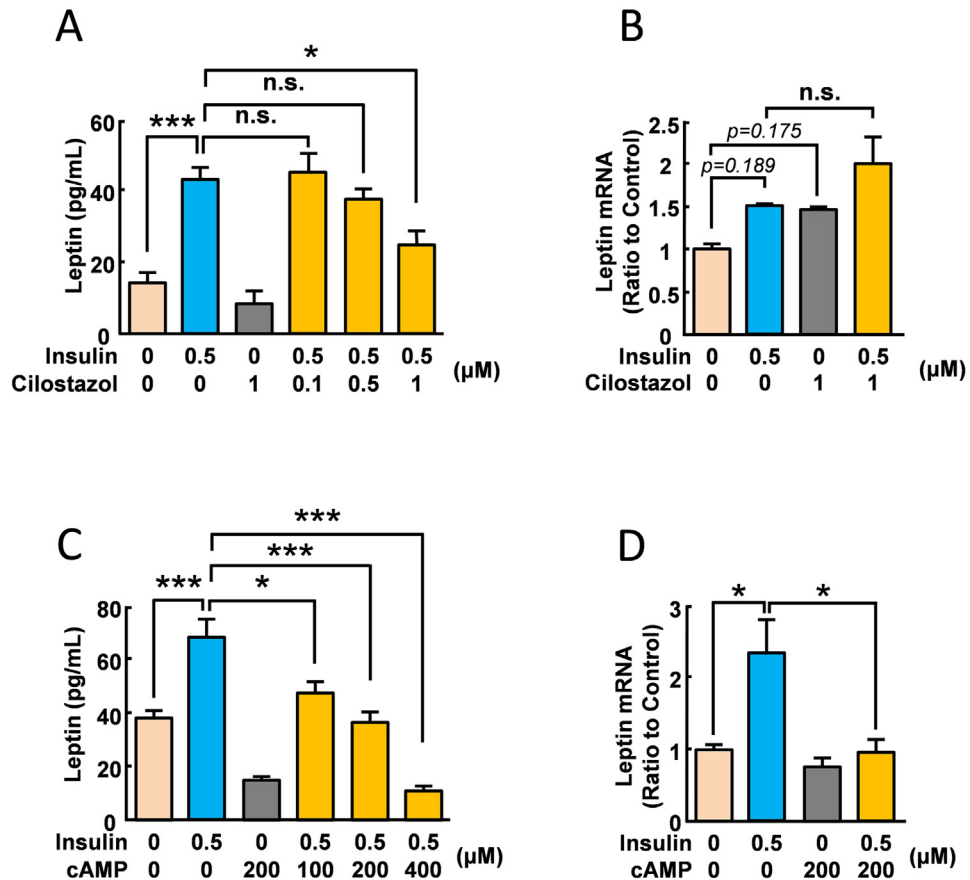


**Fig. 2.** Regulation of leptin secretion and mRNA levels by the insulin signaling pathway in 3T3-L1 adipocytes under glucose depletion. 3T3-L1 adipocytes were incubated for 8 h in glucose-free DMEM containing insulin (0.5  $\mu$ M) with or without the PI3K inhibitor BEZ-235 (0.1, 1, 10  $\mu$ M) and the Akt inhibitor MK-2206 (0.1, 1, 10  $\mu$ M). (A and B) Effects of BEZ-235, (C) wortmannin, and (D and E) MK-2206 on leptin secretion and mRNA expression. (F) MTS assay after 24 h-incubation. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . n.s., not significant. Data are expressed as means  $\pm$  SE.

(Fig. 3A and B). These effects were similar to those of the PI3K inhibitor and the Akt inhibitor. A cAMP analog, dibutyryl cAMP, also decreased leptin secretion (Fig. 3C). In contrast to cilostazol, the insulin upregulation of leptin mRNA was abrogated by dibutyryl cAMP (Fig. 3D).

### 3.4. Epinephrine decreases leptin secretion and mRNA

Since cAMP acts as the second messenger in the signal transmission of multiple hormones including epinephrine, we next examined the effect of epinephrine on leptin secretion and expression. Epinephrine dose-dependently decreased leptin secretion from adipocytes cultured with glucose-free medium (Fig. 4A). Concomitantly, the mRNA expression of leptin was reduced by epinephrine in a dose-dependent manner (Fig. 4B). The epinephrine suppression of leptin secretion was not improved with prazosin, an  $\alpha$ 1-adrenergic receptor antagonist, but significantly ameliorated with propranolol, a nonselective beta-adrenergic receptor antagonist (Fig. 4C). In harmony, propranolol partially, though statistically insignificant, recovered the epinephrine suppression of leptin mRNA (Fig. 4D). Epinephrine clearly inhibited insulin-induced leptin hypersecretion (Fig. 4E).

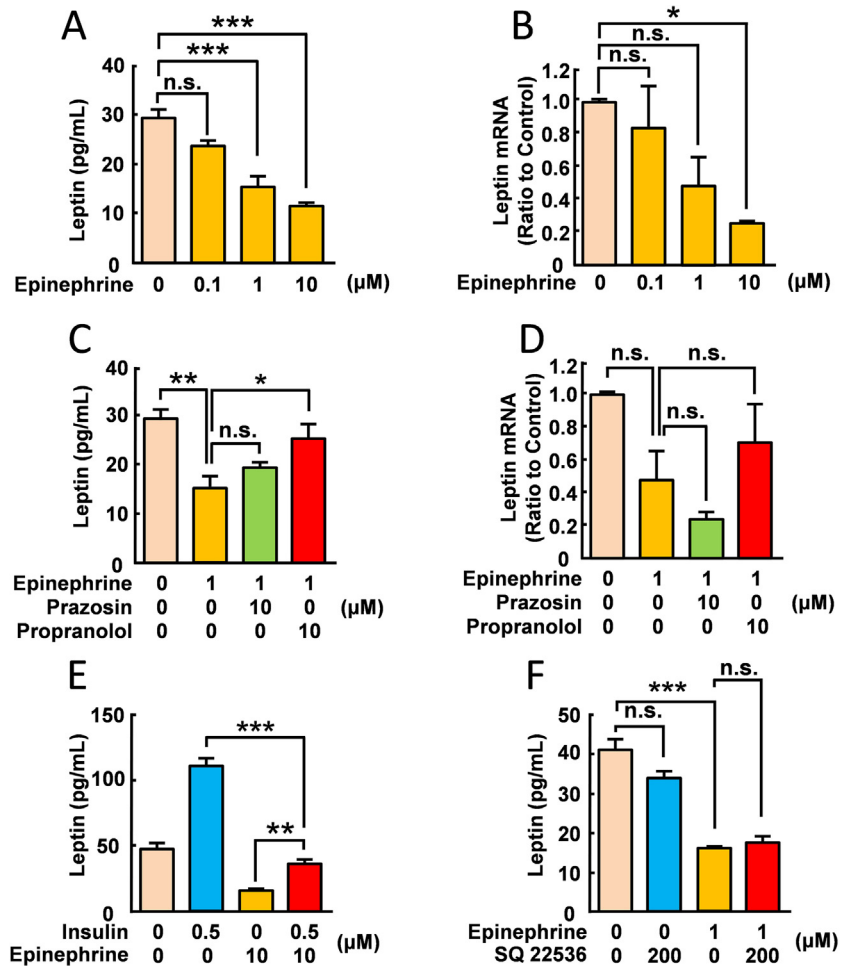


**Fig. 3.** Effects of the PDE3B inhibitor cilostazol (A and B) or a cyclic AMP analog (C and D) on insulin-induced leptin secretion and mRNA levels in 3T3-L1 adipocytes under glucose-free conditions. 3T3-L1 adipocytes were incubated for 8 h in glucose-free DMEM with each substance at the indicated concentrations. \* $p < 0.05$ , \*\*\* $p < 0.001$ . n.s., not significant. Data are expressed as means  $\pm$  SE.

Unexpectedly, an adenylate cyclase inhibitor, SQ22536, failed to significantly attenuate the epinephrine suppression of leptin secretion (Fig. 4F).

### 3.5. Regulation of leptin secretion and mRNA expression by insulin signaling in the presence of glucose

We also examined the regulation of leptin secretion and mRNA expression by insulin signaling in 3T3-L1 adipocytes under normal glucose levels. Insulin-induced leptin secretion was blocked by both the PI3K inhibitor and the Akt inhibitor (Fig. 5A). In contrast to the glucose-free condition, leptin mRNA expression was decreased by the PI3K and Akt inhibitors in the normal glucose condition (Fig. 5B), despite the fact that insulin did not augment leptin expression in the presence of glucose. A PDE3B inhibitor, cilostazol, failed to suppress insulin-induced leptin secretion in the presence of glucose (Fig. 5C). Both leptin secretion and mRNA levels were also reduced by dibutyryl cAMP in the presence



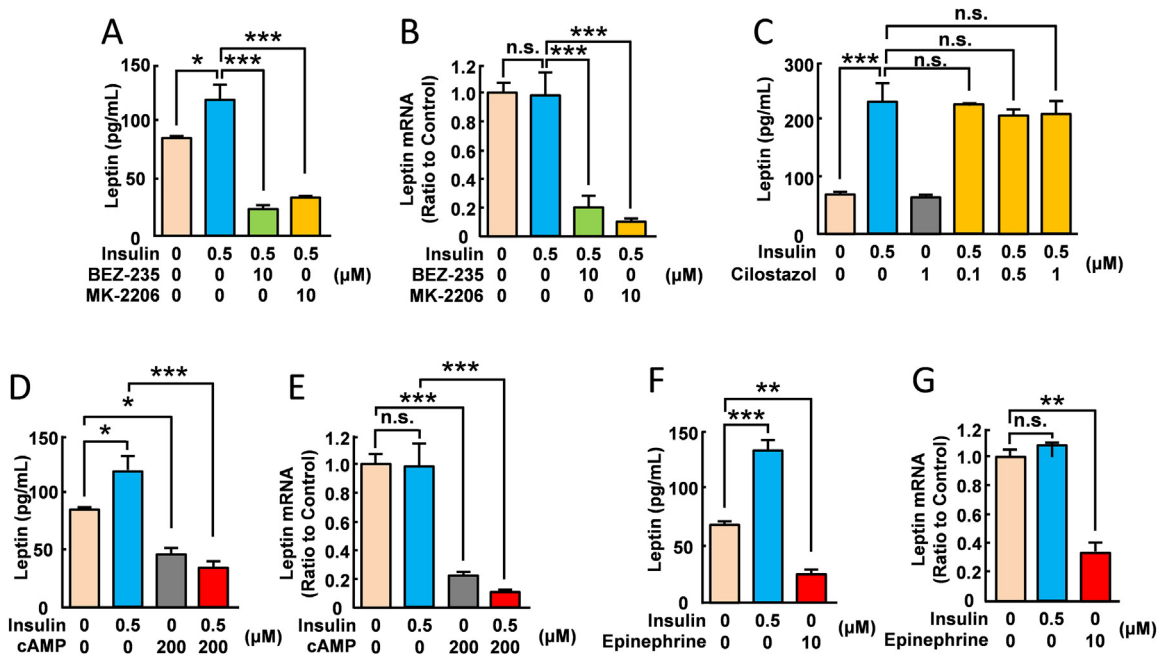
**Fig. 4.** Effect of epinephrine on leptin secretion and mRNA levels under glucose depletion. 3T3-L1 adipocytes were incubated for 8 h in glucose-free DMEM with epinephrine, an alpha1-adrenergic receptor antagonist (prazosin), and a nonselective beta-adrenergic receptor antagonist (propranolol). (A and B) Effects of epinephrine on leptin secretion and mRNA levels. (C and D) Effects of prazosin and propranolol on epinephrine-induced suppression of leptin secretion and mRNA levels. (E) Epinephrine suppresses insulin effects on leptin secretion. (F) Effect of an adenylate cyclase inhibitor, SQ22536, on the epinephrine suppression of leptin. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . n.s., not significant. Data are expressed as means  $\pm$  SE.

of glucose, as was observed under glucose depletion (Fig. 5C and D). Epinephrine showed similar results as the cAMP analog did (Fig. 5E and F).

#### 4. Discussion

In this study, we found that insulin increased leptin secretion from 3T3-L1 adipocytes under the complete depletion of glucose. The glucose level in the medium did not affect leptin secretion after insulin stimulation, while basal leptin secretion was elevated along with increasing glucose levels. Our results also showed that inhibitors for PI3K and Akt were able to suppress the insulin





**Fig. 5.** Effects of the PI3K inhibitor BEZ-235 and the Akt inhibitor MK-2206 (A and B), cilostazol (C), cAMP (D and E), and epinephrine (F and G) on leptin secretion and mRNA levels in 3T3-L1 adipocytes in the presence of 5.5 mM glucose with insulin. 3T3-L1 adipocytes were incubated for 8 h in DMEM containing 5 mM glucose with a combination of the substances at the indicated concentrations. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ . n.s., not significant.

stimulation of leptin secretion in glucose-free media. These data, consistent with previous studies [15, 16, 17], indicate that insulin alone enhances leptin secretion and accelerated glucose uptake and catabolism are unlikely to be essential to the insulin effects on leptin. It was previously reported that insulin had no effect on leptin secretion from primary adipocytes under glucose-free condition [22], although that study was performed using the Krebs-Ringer buffer deprived of not only glucose but also of amino acids and other nutrients, differing from our glucose-free DMEM containing various amino acids. Indeed, the addition of amino acids, but not glucose, recovered insulin action on leptin in the study [22].

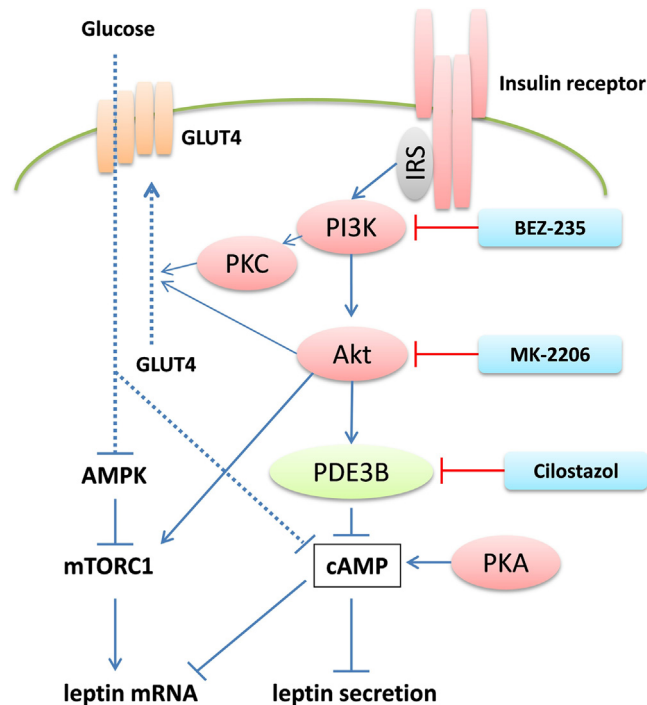
We also demonstrated that a PDE3B inhibitor and a cAMP analog abrogated insulin stimulation of leptin secretion under glucose depletion. In addition, epinephrine markedly inhibited leptin secretion from adipocytes, probably through  $\beta$ -adrenergic receptors. Previous studies performed with normal glucose levels have shown that insulin and  $\beta$ -adrenergic agonists affect leptin secretion via cAMP-dependent mechanisms [7, 19]. These data strongly suggest that cAMP reduction as a result of augmented insulin signaling plays a central role in the insulin-induced leptin secretion, independent of glucose metabolism.

On the contrary, we found that basal leptin secretion without insulin stimulation was associated with glucose levels in the media. A previous study reported that 2-deoxy-D-glucose (2DG), a glucose analog that inhibits glucose catabolism, attenuated glucose-induced leptin secretion [27]. In addition, pyruvate and gluconeogenic amino acids potently stimulated leptin secretion in the absence of insulin or ambient glucose [22]. These data indicate that glucose metabolism is also involved in leptin secretion. Because the glucose-induced leptin secretion was not observed under insulin stimulation, there seems to be an interaction between glucose and insulin. Glucose is known to inhibit adenylate cyclase and subsequently decreases intracellular cAMP levels [28, 29]. This effect likely intertwines the insulin suppression of cAMP and thus may explain why glucose and insulin do not show an additional effect on leptin secretion. Our data that a PDE3B inhibitor lost suppressive effects on leptin secretion in the presence of glucose could be relevant to the cAMP levels.

It should be noted that there is an overt discrepancy between the secretion and mRNA levels of leptin in the response to insulin, glucose and inhibitors for the PI3K-Akt pathway. In contrast to secretion, leptin mRNA levels tended to be suppressed in the presence of glucose and the insulin-induced mRNA increase was abolished by glucose. Inhibitors for PI3K-Akt signaling suppressed insulin-induced leptin secretion, but not leptin mRNA in a glucose-free condition. Intriguingly, our data showed that the inhibitors for PI3K and Akt suppressed leptin mRNA in the presence of glucose, despite the fact that insulin failed to augment leptin mRNA

It has been reported that leptin is stored mainly as a membrane-bound form in adipocytes and insulin enhances leptin secretion, but not leptin synthesis [30]. In addition, insulin was reported to increase leptin synthesis without affecting its mRNA levels [31]. These findings along with present data indicate that leptin secretion is regulated by mechanisms independent of its mRNA levels.

Although detailed mechanisms for leptin secretion and synthesis remain to be elucidated, the AMPK-mTOR system, a mechanism related to intracellular energy status, may affect insulin-induced leptin synthesis (Fig. 6). Insulin stimulates mTORC1, which possibly enhances the translation of leptin mRNA [32] and regulates promoter activity for leptin genes [33]. These observations appear relevant to the present results that insulin lost its stimulatory effects on leptin mRNA under glucose-sufficient condition, where mTORC1 is already activated as a result of suppressed AMPK [34]. In addition, the low level of cAMP in the presence of glucose may cause AMPK inactivation, because cAMP has been reported to activate AMPK in adipocytes [35]. In harmony, our data clearly showed that the direct addition of a cAMP analog, in contrast to insulin of which effect on cAMP is likely influenced by glucose, suppressed leptin mRNA levels regardless of whether glucose is present. However, this AMPK-mTOR hypothesis



**Fig. 6.** Hypothetical explanation for insulin-glucose interaction in leptin regulation in adipocytes.

may be vulnerable because our data showed that basal leptin mRNA levels were not increased but rather decreased by the presence of glucose.

It also remains to be clarified why the inhibitors for PI3K and Akt suppressed leptin mRNA only in the presence of glucose, where insulin failed to augment leptin mRNA. Although the reason is unknown, the PI3K-Akt pathway mediates signals from multiple receptors other than insulin and thus hypothetically affects leptin mRNA levels independent of insulin action

Our data have shown that cAMP rather than glucose metabolism plays a primary role in the insulin effect on leptin, indicating that hormones activating adenylate cyclase may directly suppress the insulin action on leptin. Indeed epinephrine strongly suppressed the insulin action on leptin in the present study regardless whether or not glucose is present, although it has to be explained why an adenylate cyclase inhibitor failed to ameliorate the epinephrine effect. Because epinephrine along with glucocorticoids and glucagon has been reported to increase in obesity and metabolic syndrome [36, 37, 38], these adenylate cyclase stimulating hormones possibly diminish the leptin elevation in obesity and exacerbate the pathophysiology of the disease.

It should be noted that the interpretation of present results is limited because this study lacks *in vivo* experiments and 3T3-L1 adipocytes, though most established cell lines, have been reported to show some different characteristics from primary

adipocytes, including low leptin secretion [5]. The detailed mechanisms of leptin regulation such as trafficking, transcription, translation and degradation also remain to be elucidated.

## 5. Conclusion

Insulin regulates leptin secretion and mRNA expression in adipocytes via cAMP. Glucose metabolism is not essential for the insulin effects but may indirectly influence the insulin effects, speculatively through altered cAMP levels. Given the importance of leptin in the pathogenesis of obesity and related diseases, detailed regulatory mechanisms of the hormone need further clarification.

## Declarations

### Author contribution statement

Tomomi Tsubai: Performed the experiments; Wrote the paper.

Yukihiro Noda, Makoto Nakao, Yusuke Seino: Analyzed and interpreted the data.

Kazuma Ito: Contributed reagents, materials, analysis tools or data.

Yutaka Oiso: Wrote the paper.

Yoji Hamada: Conceived and designed the experiments; Wrote the paper.

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## Competing interest statement

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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